Chemotherapy-Educated Macrophages Promote Dormant Tumor Cell Reactivation by Shaping a Pro-Fibrotic Niche

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Abstract:

Recurrence of metastatic breast cancer (BC) after neoadjuvant chemotherapy remains a major clinical challenge and is often driven by dormant disseminated tumor cells (DTCs) that escape initial treatment and later reawaken in distant organs. While cytotoxic therapies target proliferating tumor cells, they may also alter the tumor microenvironment in ways that promote metastatic progression. However, the mechanisms linking chemotherapy to DTCs reactivation remain poorly understood. We hypothesized that macrophages engulfing chemotherapy-treated tumor cells are reprogrammed into a pro-metastatic phenotype, secreting factors that contribute to the formation of a fibrotic niche permissive to DTCs outgrowth. To test this, we performed RNA sequencing on macrophages that had phagocytosed Her2+ breast cancer cells (D2A1 cells) pre-treated with either doxorubicin (DOX) or paclitaxel (PTX). Transcriptional profiling revealed distinct signatures: DOX-exposed macrophages (Mφ-DOX) upregulated immune defense genes, whereas PTX-exposed macrophages (Mφ-PTX) showed a strong pro-fibrotic signature. Both macrophage populations shared enrichment in pathways associated with extracellular matrix remodeling, angiogenesis, and wound healing, hallmarks of metastatic niche activation. Secreted mediators of Mφ-DOX and Mφ-PTX promoted the escape of dormant DTCs from quiescence in a 3D dormancy model and in vivo. Previously, we demonstrated that a fibrotic niche supports DTCs outgrowth. Likewise, our results suggest that secreted mediators of these chemo-educated macrophages contribute to fibrotic niche formation, accelerating DTCs reactivation via beta-1 integrin.

Significance: These findings uncover a novel mechanism by which chemotherapy may inadvertently promote recurrence by educating macrophages into pro-metastatic effectors. Identifying the pro-metastatic mediators secreted by these chemo-educated macrophages may uncover novel therapeutic targets. This approach holds promise for developing complementary treatments to prevent BC recurrence and improve long-term patient outcomes.

Hypothesis: Macrophages (Mφ) engulfing chemotherapy eradicated metastatic cells are reprogrammed to promote a permissive microenvironment for dormant DTCs outgrowth

Results:

Experimental design

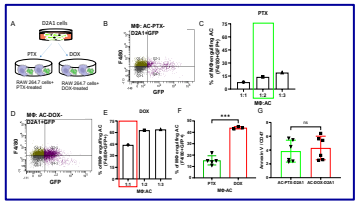
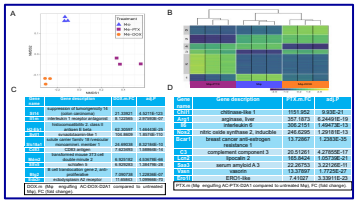


Figure 1: A) Scheme demonstrating the preparation of Mφ engulfing DOX/PTX treated D2A1-GFP cells and their respective conditioned media. **B-F)** Mφ were incubated with apoptotic D2A1-GFP cells (green) at the indicated ratios respectively. Apoptotic D2A1 cells were obtained by treatment either with PTX (800nM) (AC-PTX-D2A1) (B-C) or DOX (10μM) (AC-DOX-D2A1) (D-E). **B and D)** Representative Dot plot of F4/80 and GFP staining of Mφ engulfing AC. **C and E)** Quantification of the percentage of Mφ engulfing either AC-PTX-D2A1 cells (C) or AC-DOX-D2A1 (E) (n=1). **F)** Quantification of the percentage of Mφ engulfing either AC-PTX-D2A1 cells (n=5) or AC-DOX-D2A1 (n=3). **G)** Ratio of annexinV/CD47 expression on AC-PTX-D2A1 or AC-DOX-D2A1. n=6. Columns; mean, bars; STD. ***p≤0.001.

Transcriptome analysis of macrophages engulfing DOX/PTX-treated D2A1 cells points to distinct identity



Gene	Gene description	FC PTX/M	adj.P
Agrn1	chitinase-like 1	1151.952	9.93E-21
Agrn1	arginase, liver	357.1873	6.24491E-19
Il6	interleukin 6	306.2151	1.49473E-15
Nos2	nitric oxide synthase 2, inducible	246.6295	1.29181E-16
Spp1	secreted phosphoprotein 1	13.72867	1.23883E-10
Il1rn	interleukin 1 receptor antagonist	11.83621	6.52158E-12
C3	complement component 3	20.51261	4.27855E-17
Ccl22	chemokine (C-C motif) ligand 22	165.8424	1.05729E-20
Saa3	serum amyloid A 3	22.26753	3.22129E-11
Vsig4	V-set and immunoglobulin domain containing 4	13.37697	1.77268E-07
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DOX vs. Mφ engulfing AC-DOX-D2A1 (compared to untreated Mφ), FC, DM changes

PTX vs. Mφ engulfing AC-PTX-D2A1 (compared to untreated Mφ), FC, DM changes

Figure 2: A) Non-metric multidimensional scaling analysis (NMDS) of whole-transcriptome RNA-seq data of untreated macrophages (Mφ) and macrophages engulfing either PTX-treated D2A1 cells (Mφ-PTX) or DOX treated D2A1 cells (Mφ-DOX) (based on Bray-Curtis dissimilarities, K=2, stress=0.033). **B)** Heatmap of differentially expressed genes between Mφ and Mφ-PTX or Mφ-DOX. Among 15,282 expressed coding genes, 12,620 genes were differentially expressed in at least one pair of comparisons (adj. P-value <0.05) as determined by DESeq2 analysis. Values displayed correspond to the z-scaled expression level for each gene. Vertical clusters were calculated by K-means where K=6. **C-D)** Selected genes that are exclusively and differentially expressed only by Mφ engulfing DOX-treated D2A1 cells (AC-DOX-D2A1) (C) or by Mφ engulfing PTX treated D2A1 cells (AC-PTX-D2A1) (D) FC ≥2, adj.P (adjusted P values) ≤0.05 compared to untreated Mφ.

Transcriptome of macrophage engulfing DOX/PTX-treated D2A1 cells points to M1/M2 mixed signature and remodeling of ECM

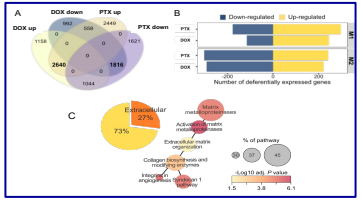


Figure 3: A) Venn diagram depicting the number of differentially transcribed genes (Benjamini-Hochberg adj. P<0.05) in Mφ engulfing D2A1 cells treated with DOX /PTX. **B)** Transcription patterns of M1 and M2 Mφ signature *in vitro* (Orecchioni et al., 2019) in Mφ engulfing D2A1 cells treated with DOX/PTX. **C)** Functional gene set enrichment analysis (ENRICHR, https://maayanlab.cloud/Enrichr/;accessed May, 2022) for common up-regulated genes (against the BioPlanet database). Shown are functional gene sets related to extracellular matrix remodeling.

Macrophage engulfing DOX/PTX-treated D2A1 cells express pro-metastatic genes

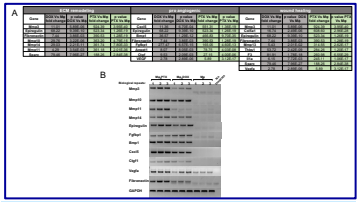


Figure 4: A) Selected ECM remodeling, pro-angiogenic and wound healing genes expressed by Mφ engulfing PTX/DOX-treated D2A1 cells. Fold change ≥2, p. values ≤0.05. **B)** Semi-qPCR of selected ECM remodeling pro-angiogenic and wound healing genes expressed by both Mφ engulfing PTX/DOX-treated D2A1 cells (Mφ-PTX or Mφ-DOX, respectively) compared to untreated Mφ.

Soluble mediators generated by macrophages engulfing either DOX/PTX-treated D2A1 cells induce dormant D2A1 cell outgrowth *in vivo*

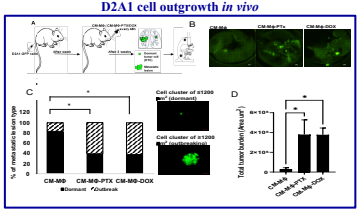


Figure 5: A) Scheme of experimental design *in vivo*. **B)** Representative single cell organ microscopy (SCOM) images of the whole lungs treated with either CM-Mφ, CM-Mφ-PTX or CM-Mφ-DOX. Magnification x10, n=8. **C)** Left panel: Percentage of dormant single cells versus multi-cellular proliferative metastatic lung lesions in mice. Right panel: Representative images of single cells (foci ≤1200 μm2) and multi-cellular proliferative metastatic lung lesions (cell clusters >1200 μm2). **D)** Total D2A1-GFP tumor cell burden/lung. n=8 mice; Columns; mean, bars; STE, * p≤0.05.

Fibrotic-like milieu at the metastatic site in mice treated with CM-Mφ PTX/DOX

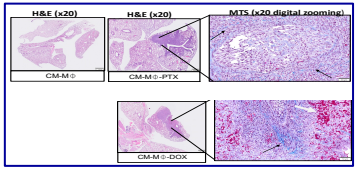
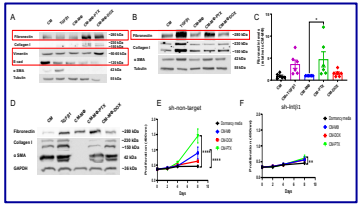


Figure 6: Representative paraffin sections (n=3) of lungs colonized with D2A1-GFP cells treated with the different conditioned media. Paraffin sections were subjected to either hematoxylin and eosin staining (H&E) or type I collagen (Col-I) expression determined by Masson's trichrome stain (MT;blue) (see black arrow). White arrow denotes angiogenesis.

CM-Mφ PTX/DOX promotes epithelial to mesenchymal transition (EMT) of D2A1 cells which in turn promotes lung fibroblasts activation and D2A1 cells outgrowth *in vitro*



Summary:

Macrophages (Mφ) that engulf DOX/PTX-treated D2A1 metastatic breast cancer cells are reprogrammed into distinct phenotypes and express pro-metastatic genes. Their conditioned media (CM-Mφ-DOX/PTX) promotes metastatic outgrowth of dormant D2A1 cells *in vitro* and *in vivo* by inducing fibronectin expression in lung fibroblasts and tumor cells, which drives outgrowth via Intβ1. Notably, Mφ from PTX-treated breast cancer patients exhibit a similar pro-fibrotic signature, highlighting the clinical relevance of these findings.

Figure 7: A) Representative W.B. of EMT markers in D2A1 cells treated with TGFβ1, CM, CM-Mφ, CM-Mφ-PTX, or CM-Mφ-DOX. **B)** Representative W.B. of lung fibroblasts (Mlg) activation under the same treatments. **C)** Fibronectin quantification; normalized to CM-Mφ. Columns; mean, bars; STE, n=6. *P≤0.05. **D)** Representative W.B. of fibronectin, Col-I and α-SMA expression in Mlg cells either treated with TGFβ1 (positive control) or treated with conditioned media derived from D2A1 cells that were treated for 48h with CM, CM-Mφ, CM-Mφ-PTX or CM-Mφ-DOX. **E-F)** Proliferation of D2A1-sh-non-target and of D2A1-sh-Intβ1 cells cultured in the 3D BME system treated with the indicated conditioned media. n=5, One-way repeated measures ANOVA analysis with repeated contrasts. **** p<0.0001, ** p≤0.01, *p ≤ 0.05.

Tumor-infiltrating macrophages of TNBC patients, before and after PTX treatment, exhibit a pro-inflammatory and pro-fibrotic predicted secretome.

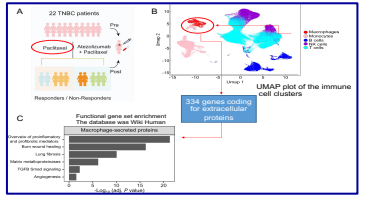


Figure 8: A) Single cell transcriptome analysis design and data from Zhang Y et al. (2021). **B)** Umap landscape of immune cells for the subset of PTX-treated patients. Major annotated cell clusters are highlighted by colors. **C)** Functional gene-set enrichment analysis of predicted secretome of macrophages. Expression of 334 genes coding for extracellular proteins was detected in tumor-associated macrophages. Functional gene-set enrichment analysis for these genes was carried out against the Wiki-Human database. Enrichment was considered significant for FDR adjusted P value < 0.05.